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ORIGINAL REPORT

# Intratumoral Heterogeneity of *ALK*-Rearranged and *ALK*/*EGFR* Coaltered Lung Adenocarcinoma

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## ABSTRACT

### Purpose
Genetic intratumoral heterogeneity has a profound influence on the selection of clinical treatment strategies and on addressing resistance to targeted therapy. The purpose of this study was to explore the potential effect of intratumoral heterogeneity on both genetic and pathologic characteristics of *ALK*-rearranged lung adenocarcinoma (LADC).

### Methods
We tested *ALK* fusions and *EGFR* mutations in 629 patients with LADC by using laser-capture microdissection to capture spatially separated tumor cell subpopulations in various adenocarcinoma subtypes and to test for *ALK* fusions and *EGFR* mutations in *ALK*-rearranged, *EGFR*-mutated, and *ALK*/*EGFR* coaltered LADCs to compare the oncogenic driver status between different tumor cell subpopulations in the same primary tumor.

### Results
Among the 629 patients, 30 (4.8%) had *ALK* fusions, 364 (57.9%) had *EGFR* mutations, and two had *ALK* fusions that coexisted with *EGFR* mutations. Intratumoral heterogeneity of *ALK* fusions were identified in nine patients by reverse-transcriptase polymerase chain reaction. In the two patients with an *ALK*/*EGFR* coaltered status, genetic intratumoral heterogeneity was observed both between different growth patterns and within the same growth pattern. The relative abundance of *ALK* and *EGFR* alterations was different in the same captured area. *ALK* fusions were positively associated with a micropapillary pattern ($P = .002$) and were negatively associated with a lepidic pattern ($P = .008$) in an expanded statistical analysis of 900 individual adenocarcinoma components, although they appeared to be more common in acinar-predominant LADCs in the analysis of 629 patients.

### Conclusion
Intratumoral genetic heterogeneity was demonstrated to coexist with histologic heterogeneity in both single-driver and *ALK*/*EGFR* coaltered LADCs. Altered oncogenic drivers in spatially separated subclones of the same tumor may be different.

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## INTRODUCTION

Tumor heterogeneity is currently a topic of great interest in cancer research, because it poses a series of challenges to both accurate diagnosis and personalized therapy. Morphologic heterogeneity has long been recognized and forms the basis of many tumor grading prognostic classification systems. In recent years, genetic heterogeneity has been demonstrated in malignant tumors, including breast cancer, leukemia, renal cancer, medulloblastoma, and non–small-cell lung cancer (NSCLC).[1-5] Furthermore, with the development of gene detection technology, genetic heterogeneity has been identified not only between individual tumors of the same histopathologic subtype but also between primary lesions and associated metastatic sites in the same patient,[3,6] and even between spatially separated regions within single biopsies obtained from a primary tumor.[7,8] Because most clinical decision making for patients with advanced NSCLC depends on single tumor biopsy samples obtained from primary or metastatic sites, and because the tumor genomics landscape portrayed from single tumor biopsy samples may be inaccurate and underestimated,[3] intratumoral genetic heterogeneity is a focus of attention of investigators from lung cancer fields.

In clonal evolution models, genetic diversity may occur in regionally separated regions within a primary

tumor through branched evolutionary tumor growth.[7,8] However, for lung adenocarcinoma (LADC) with a high degree of morphologic heterogeneity,[9] the relationship between intratumoral genetic heterogeneity demonstrated in spatially separated regions within the same primary LADC lesion and its histologic diversity remains unclear. Although the histopathologic features of *ALK* rearrangements were reported in several studies,[10-16] these studies did not make much sense as expected, because they did not fully realize the potential impact of both the genetic and morphologic intratumoral heterogeneity of LADC. Therefore, in this study, laser-capture microdissection (LCM) was used to capture pure tumor cells within the same growth pattern, and the correlation of *ALK* fusions with pathologic features was analyzed in 900 individual adenocarcinoma components to control for histologic heterogeneity to a large extent.

In addition, on the basis of the trunk-branch clonal evolution hypothesis, genetic aberrations present in the trunk may be ubiquitous mutations, whereas those in the branch may be heterogeneous mutations in a tumor.[17] For tumors that harbor dual oncogenic drivers concurrently, whether one is ubiquitous or both are ubiquitous remains unknown. In our study, we used LCM to capture spatially separated tumor cell subpopulations according to adenocarcinoma subtypes, and we tested *ALK* fusions and *EGFR* mutations, respectively, to explore the potential mechanism of intratumoral heterogeneity and provide evidence for selecting targeted therapy in patients who have NSCLC with *ALK*/*EGFR* coalterations.

## METHODS

### *Patients and Tissue Samples*

Formalin-fixed and paraffin-embedded (FFPE) tissue sections were obtained from patients with histologically confirmed primary LADC who underwent surgical resection at Shanghai Pulmonary Hospital between 2004 and 2010. Pathologic diagnosis and staging were performed according to the 2011 International Association for the Study of Lung Cancer (IASLC)/American Thoracic Society (ATS)/European Respiratory Society (ERS) International Multidisciplinary Classification of Lung Adenocarcinoma and the TNM staging system of the IASLC, version 7. All FFPE tissue sections were reviewed by two pathologists for confirmation of the histology and assessment of the tumor content.

The study was approved by the institutional review boards of the Shanghai Pulmonary Hospital. The inclusion criteria of this study are listed in the Appendix (online only).

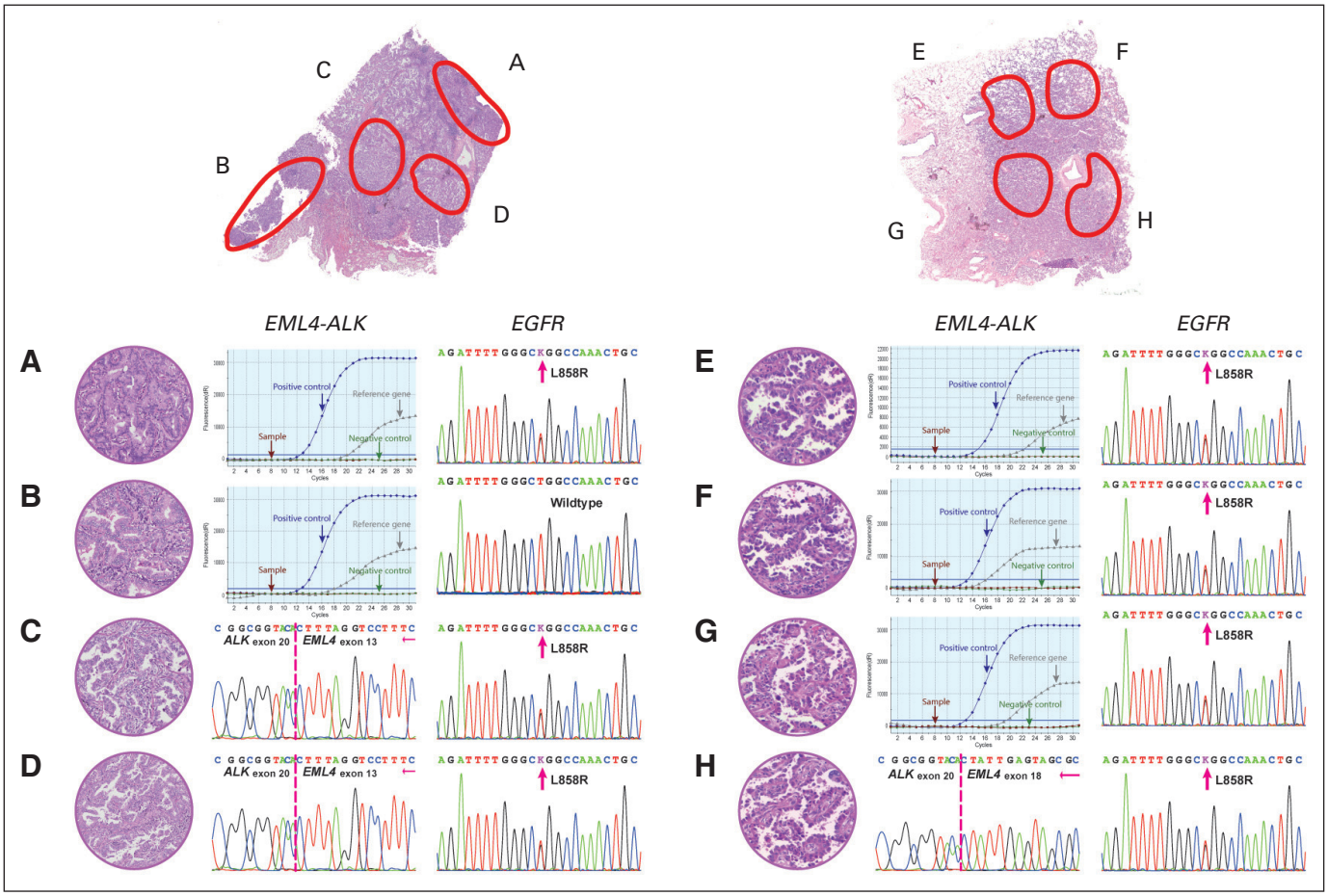


**Fig 1.** Pathologic and genetic characteristics of two patients with *ALK*/*EGFR* coaltered adenocarcinoma by reverse-transcriptase polymerase chain reaction and amplification refractory mutation system assays. (A) Acinar, positive for *EGFR* mutations (L858R) and negative for *ALK* fusions. (B) Acinar, negative for both *EGFR* mutations and *ALK* fusions. (C) Micropapillary, positive for both *EGFR* mutations (L858R) and *ALK* fusions (E13;A20). (D) Micropapillary, positive for both *EGFR* mutations (L858R) and *ALK* fusions (E13;A20). (E) Lepidic, negative for *ALK* fusions and positive for *EGFR* mutations (L858R). (F) Lepidic, negative for *ALK* fusions and positive for *EGFR* mutations (L858R). (G) Papillary, negative for *ALK* fusions and positive for *EGFR* mutations (L858R). (H) Papillary, positive for both *ALK* fusions (E18;A20) and *EGFR* mutations (L858R).

  

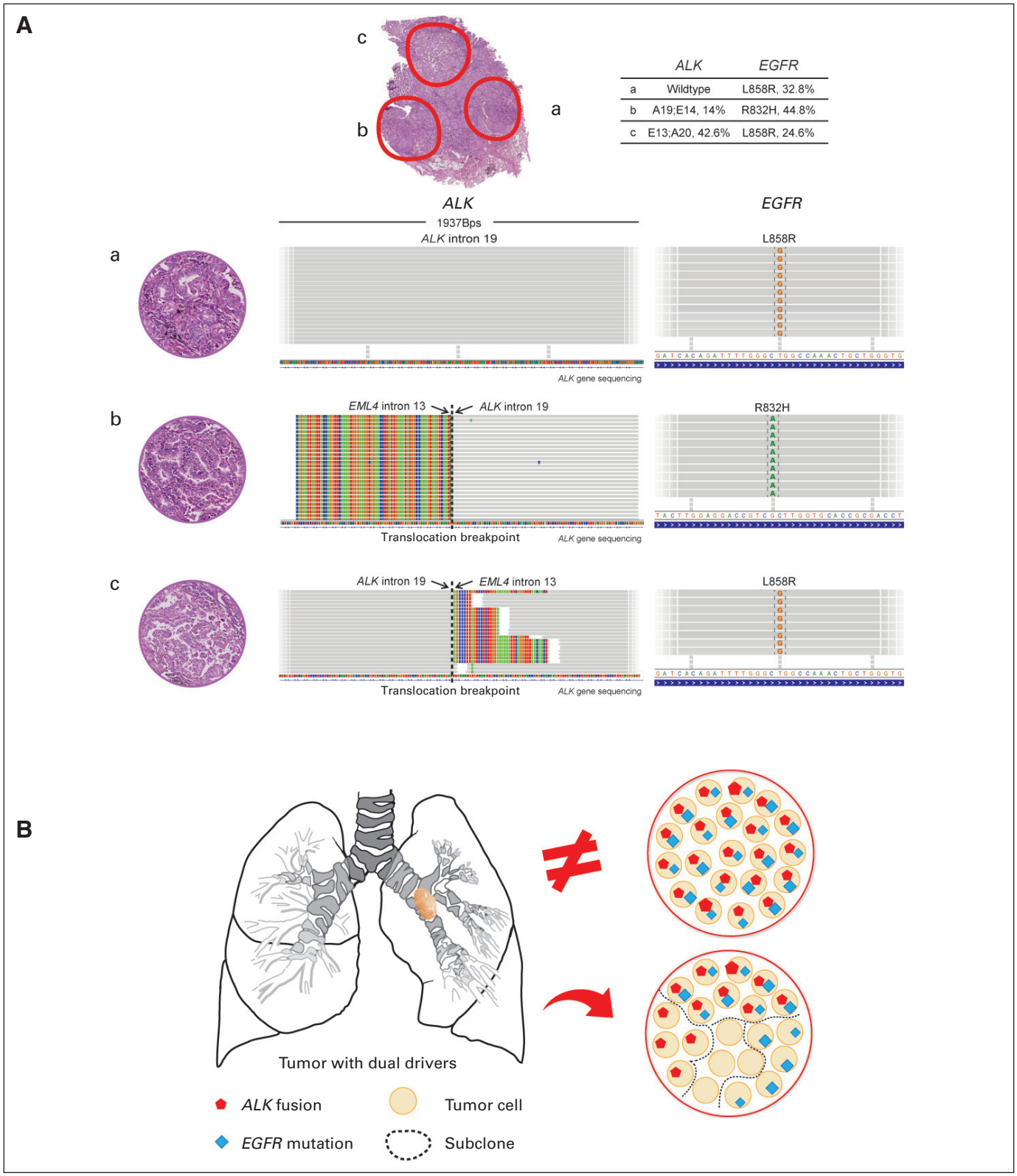


**Fig 2.** (A) Pathologic and genetic characteristics of a patient with *ALK*/*EGFR* coaltered adenocarcinoma by targeted DNA sequencing. (Aa) Acinar, *EGFR* mutations (L858R) and *ALK* wild type. (Ab) Acinar, *EGFR* mutations (R832H) and *ALK* fusions (A19;E14). (Ac) Micropapillary and papillary, *EGFR* mutations (L858R) and *ALK* fusions (E13;A20). (B) The driver status may be different among different tumor subclones from *ALK*/*EGFR* coaltered lung adenocarcinoma. These graphs show the corresponding BAM views of the *ALK* intron region around the imputed breakpoint in Integrative Genomics Viewer genome browser. Loci that match with the reference genome are shown as gray, whereas mismatching loci are shown in different colors according to the actual genotype called (adenine, green; cytosine, blue; guanine, yellow; thymine, red). The mismatched parts of the reads come from the translocation partner *EML4*. Therefore, the point between the matching and mismatching regions demonstrates the translocation breakpoint.

**Table 1.** Molecular and Pathologic Features of the 20 *ALK*-Positive Patients With LADC by RT-PCR Assay

| Isolated Area | LADC Subtype (%) | Mucin Production | *ALK* Fusion |
|---|---|---|---|
| LUC1 | | | |
| A | Acinar (90) | Yes | E13;A20 |
| B | Micropapillary (10) | Yes | Wild type |
| LUC2 | | | |
| A | Acinar (90) | Yes | E2;A20 |
| B | Micropapillary (10) | No | E2;A20 |
| LUC3 | | | |
| A | Acinar (85) | No | E18;A20 |
| B | Papillary (15) | No | Wild type |
| LUC4 | | | |
| A | Papillary (75) | No | E18;A20 |
| B | Lepidic (25) | No | Wild type |
| LUC5 | | | |
| A | Papillary (60) | No | E6;A20 |
| B | Acinar (40) | No | E6;A20 |
| LUC6 | | | |
| A | Acinar (55) | Yes | E6;A20 |
| B | Micropapillary (35) | Yes | E6;A20 |
| C | Papillary (10) | Yes | Wild type |
| LUC7 | | | |
| A | Acinar (90) | Yes | E6;A20 |
| B | Papillary (10) | Yes | E6;A20 |
| LUC8 | | | |
| A | Acinar (80) | No | E13;A20 |
| B | Lepidic (15) | No | Wild type |
| C | Papillary (5) | No | Wild type |
| LUC9 | | | |
| A | Acinar (85) | No | E6ins33;ins18A20 |
| B | Micropapillary (10) | No | E6ins33;ins18A20 |
| C | Papillary (5) | No | Wild type |
| LUC10 | | | |
| A | Acinar (80) | Yes | E13;A20 |
| B | Solid (20) | No | Wild type |
| LUC11 | | | |
| A | Micropapillary (50) | Yes | E20;A20 |
| B | Papillary (35) | Yes | E20;A20 |
| C | Acinar (15) | Yes | E20;A20 |
| LUC12 | | | |
| A | Micropapillary (60) | No | E6;A20 |
| B | Acinar (35) | No | E6;A20 |
| C | Lepidic (5) | No | Wild type |
| LUC13 | | | |
| A | Acinar (100) | Yes | E13;A20 |
| LUC14 | | | |
| A | Acinar (50) | No | Wild type |
| B | Micropapillary (40) | No | E13;A20 |
| C | Papillary (10) | No | Wild type |
| LUC15 | | | |
| A | Micropapillary (40) | Yes | E13;A20 |
| B | Acinar (30) | Yes | E13;A20 |
| C | Papillary (30) | Yes | E13;A20 |
| LUC16 | | | |
| A | Papillary (100) | No | E6;A20 |
| LUC17 | | | |
| A | Micropapillary (55) | No | E6;A20 |
| B | Papillary (35) | Yes | E6;A20 |
| C | Acinar (10) | Yes | E6;A20 |
| LUC18 | | | |
| A | Acinar (80) | No | E13;A20 |
| B | Solid (20) | No | E13;A20 |
| LUC19 | | | |
| A | Acinar (100) | Yes | E13;A20 |
| LUC20 | | | |
| A | Papillary (90) | No | E13;A20 |
| B | Micropapillary (10) | No | E13;A20 |

Abbreviations: LADC, lung adenocarcinoma; LUC, lung cancer sample; RT-PCR, reverse-transcriptase polymerase chain reaction.

### *Detection of* ALK *Fusions and* EGFR *Mutations*

All patient samples were tested for *EGFR* by using the amplification refractory mutation system (ARMS) assay with the AmoyDx *EGFR* 29 mutations detection kit and were tested for *ALK* status by using a reverse-transcriptase polymerase chain reaction (RT-PCR) assay with the AmoyDx *EML4-ALK* fusion gene detection kit (Amoy Diagnostics, Xiamen, People's Republic of China). The amplification of $\beta$-actin was used to ensure the quality of RNA extracted. The *ALK* fusion variants screened by the AmoyDx *EML4-ALK* fusion gene detection kit are shown in Appendix Table A1 (online only). All *ALK* fusion–positive or *EGFR* mutation–positive samples were validated by using direct sequencing. Details of the methodology have been described in previous studies.[18-20]

### *LCM*

All FFPE sections from *ALK* fusion–positive patients who underwent surgical resection in 2010 were stained with hematoxylin and eosin. The ArcturusXT microdissection system (Life Technologies, Carlsbad, CA) was used to capture pure cell subpopulations in target areas that were selected according to the 2011 IASLC/ATS/ERS International Multidisciplinary Classification of LADC in the *ALK*-positive samples. Greater than $10^4$ cells in each area were obtained, and one to four areas (according to the amount of tumor cells) were selectively captured in each adenocarcinoma subtype in each section. Total RNA and DNA were extracted from each captured LCM sample by using the AmoyDx FFPE DNA/RNA kit (Spin Column, ADx-FF03; Amoy Diagnostics) for all *ALK*-positive samples resected in 2010. Selected areas were tested for *ALK* fusions and *EGFR* mutations (if required) by using multiplex RT-PCR (study flow chart in Appendix Fig A1, online only).

In addition, 20 FFPE samples were randomly selected from the patients with *EGFR* mutations and microdissected according to lung adenocarcinoma histopathologic subtype. For patients with intratumoral heterogeneity of *ALK* rearrangement, 4-$\mu$m sections were recut from the same FFPE tumor tissues, and LCM was performed for targeted DNA sequencing.

### *Targeted DNA Sequencing*

For patients with available DNA, targeted DNA sequencing was performed. Genomic DNA was profiled by using a capture-based targeted sequencing panel (Burning Rock Biotech, Guangzhou, People's Republic of China). Human genomic regions of 271 kb, including all exons in 47 genes and selected introns in three of the genes for the detection of translocation events, were captured by using 120-bp probes and were sequenced (Appendix Table A2, online only). The concentration of the DNA samples was measured with the Qubit dsDNA assay to make sure that genomic DNA was greater than 40 ng. Fragments of 200 to 400-bp sizes were selected with beads (Agencourt AMPure XP kit; Beckman-Coulter, Brea, CA), followed by hybridization with the capture probes baits, hybrid selection with magnetic beads, and PCR amplification. A bioanalyzer high-sensitivity DNA assay was then used to assess the quality and size range. Available indexed samples were then sequenced on a Nextseq (Illumina, San Diego, CA) with pair-end reads. Sequence data were mapped to the human genome (hg19) with the BWA aligner

  

0.7.10 (http://bio-bwa.sourceforge.net/). Local alignment optimization, variant calling, and annotation were performed with GATK 3.2 (https://www.broadinstitute.org/gatk/). DNA translocation analysis was performed by using both Tophat2 (http://ccb.jhu.edu/software/tophat/index.shtml) and Factera 1.4.3 (http://factera.stanford.edu).[21]

### *Fluorescence in Situ Hybridization and Immunohistochemistry*

For patients with intratumoral heterogeneity of *ALK* fusions identified by RT-PCR assay, fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) assays were performed on other recut serial sections. The Vysis *ALK* breakapart FISH probe kit (Vysis/Abbott, Abbott Park, IL) was used according to the manufacturer's instructions. The presence of the breakapart probe signal in greater than 15% of tumor cells was defined as positive for *ALK* fusions. IHC was performed as a fully automated IHC assay with the prediluted Ventana (Ventana-Roche, Tucson, AZ) anti-*ALK* (D5F3) rabbit monoclonal primary antibody (Roche Diagnostics GmbH, Mannheim, Germany), Optiview DAB IHC detection kit (Ventana Medical Systems, Tucson, AZ) and Optiview amplification kit (Ventana Medical Systems) on the Benchmark XT stainer (Ventana Medical Systems). Details of assay procedures were described in the study by Wynes et al.[22] Each case was stained with a positive control and negative control. Any presence of positive staining in tumor cells was defined as positive for *ALK* fusions.

### *Statistical Analysis*

The statistical analysis was in two parts. One part was performed in 629 patients with LADC who were enrolled on the study, and the other part was performed in 900 individual adenocarcinoma components on FFPE tissue sections obtained from the 629 patients. All adenocarcinoma components quantitatively diagnosed in greater than 5% of tumor cells on FFPE tissue sections in 2010 were included in the expanded analysis, and each adenocarcinoma component was defined as an observational unit. Categoric variables were compared by using a $\chi^2$ test or Fisher's exact test. The survival curve was plotted, and the median overall survival was calculated by using the Kaplan-Meier method. The two-sided significance level was set at $P < .05$. All data were analyzed by using the Statistical Package for the Social Sciences software, version 17.0 (SPSS, Chicago, IL).

## RESULTS

### *Intratumoral Genetic Heterogeneity in* ALK/EGFR *Coaltered LADCs*

Among the 20 *ALK*-positive patients, two were found to concurrently harbor *ALK* fusions and *EGFR* mutations. For one of the two samples with *ALK/EGFR* coalteration, as shown in Figure 1, *ALK* fusions did not coexist with *EGFR* mutations in all tumor cells. Figure 1

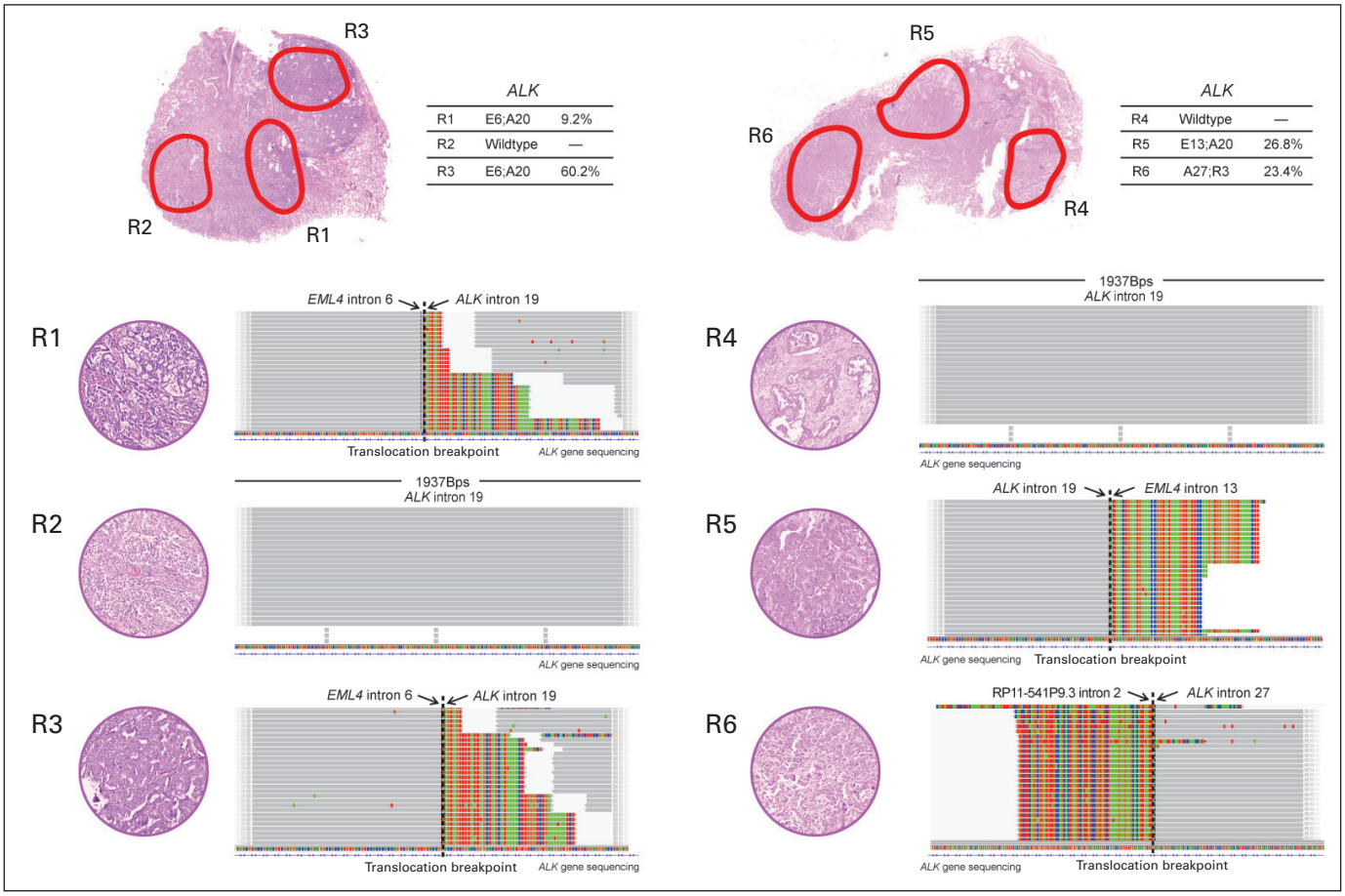


**Fig 3.** Pathologic and genetic characteristics of two patients with *ALK*-rearranged adenocarcinoma by targeted DNA sequencing. By area: R1, acinar, *ALK* fusion (E6;A20), and relative abundance of 9.2%; R2, micropapillary, *ALK* wild type; R3, acinar, *ALK* fusions (E6;A20), and relative abundance of 60.2%; R4, acinar, *ALK* wild type; R5, acinar, *ALK* fusion (E13;A20), and relative abundance of 26.8%; R6, micropapillary, *ALK* fusion (A27;R3), and relative abundance of 23.4%. These graphs show the corresponding BAM views of the *ALK* intron region around the imputed breakpoint in Integrative Genomics Viewer genome browser. Loci that match with the reference genome are shown as gray, whereas mismatching loci are shown in different colors according to the actual genotype called (adenine, green; cytosine, blue; guanine, yellow; thymine, red). The mismatched parts of the reads come from the translocation partner *EML4* or *RP11-541P9.3*. Therefore, the point between the matching and mismatching regions demonstrates the translocation breakpoint.

indicates that tumor cells in both area C and area D were of the micropapillary subtype, which were positive for both *ALK* fusion and *EGFR* mutation. Of interest, tumor cells captured in area A were negative for *ALK* fusions and positive for *EGFR* mutations, whereas tumor cells in area B were negative for both *ALK* fusions and *EGFR* mutations. Then, we used targeted DNA sequencing to confirm the intratumoral genetic heterogeneity on the recut FFPE section from the same *ALK/EGFR* coaltered tumor. We also observed similar findings, although we could not capture tumor cells according to previous regions, because tumor morphology on recut sections changed. As shown in Figure 2, tumor cells in area C were confirmed positive for both *ALK* fusion and *EGFR* mutation. Of interest, the relative abundances of *ALK* fusions and *EGFR* mutations in the same dissected region were 42.6% and 24.6%, respectively (Fig 2A). In other words, some tumor cells from this dissected region harbored only *EML4-ALK* fusions (Fig 2B). Except for the *ALK/EGFR* coaltered area, area B harbored *ALK-EML4* fusions with a low relative abundance of 14.0%, which might not be detected by cDNA-based methods. Tumor cells in area B also harbored the *EGFR* R832H mutation rather than L858R, with a relative abundance of 44.8%. The findings suggested that some tumor cells from this area were negative for both *EML4-ALK* fusions and the *EGFR* L858R mutation (Fig 2B).

For the other sample with *ALK/EGFR* coalteration, all selected areas, including papillary and lepidic patterns, were positive for *EGFR* mutations, although only area H (papillary) was positive for *ALK* fusions (Fig 1). Unfortunately, the remaining tissue was not available for targeted DNA sequencing.

### *Intratumoral Genetic Heterogeneity in* ALK*-Rearranged LADCs*

Among the 20 *ALK*-positive patients, nine patients had *ALK* wild-type regions on FFPE sections by RT-PCR (Table 1). For these nine patients, Ventana IHC and FISH were also used to confirm the intratumoral heterogeneity of *ALK* rearrangement on recut FFPE sections, although the positive regions did not fully match among the three methods (Appendix Fig A2, online only). In addition, of these nine patients, we performed targeted DNA sequencing in two of them whose samples had available DNA to confirm intratumoral genetic heterogeneity. As shown in Figure 3, both areas R1 and R3 from one tumor were positive for E6;A20, and the relative abundance was 9.2% and 60.2%, respectively. However, area R2 from the same tumor was negative for E6;20. Similar results were observed in the other tumor. Area R5 was positive for E13;A20, whereas area R4 was negative for *ALK* fusions. Furthermore, tumor cells in area R6 harbored an *ALK*-RP11-541P9.3 fusion rather than E13;A20, and the relative abundance of this fusion was 23.4% (Fig 3).

### *Intratumoral Genetic Heterogeneity in* EGFR*-Mutated LADCs*

Among the 20 patients randomly selected from those with *EGFR*-mutated tumors, genetic intratumoral heterogeneity was demonstrated in five by ARMS assay. Intratumoral genetic heterogeneity of *EGFR* mutations was identified both between different adenocarcinoma subtypes and within the same adenocarcinoma component. (Appendix Table A3, online only).

**Table 2.** Expanded Statistical Analysis of the 900 Individual Adenocarcinoma Components

| Characteristic | No. (%) of Patients: All (N = 900) | *EML4-ALK* Fusion: Positive (n = 34) | *EML4-ALK* Fusion: Negative (n = 866) | *P* |
|---|---|---|---|---|
| Mucin production | | | | < .001 |
| Yes | 139 (15.4) | 17 (50.0) | 122 (14.1) | |
| No | 761 (84.6) | 17 (50.0) | 744 (85.9) | |
| Histologic subtype | | | | |
| Lepidic | 150 (16.7) | 0 | 150 (17.3) | .008 |
| Acinar | 345 (38.3) | 16 (47.1) | 329 (38.0) | .286 |
| Papillary | 287 (31.9) | 8 (23.5) | 279 (32.2) | .286 |
| Micropapillary | 78 (8.7) | 9 (26.5) | 69 (8.0) | .002 |
| Solid | 24 (2.7) | 1 (2.9) | 23 (2.7) | .608 |
| IMA | 15 (1.7) | 0 | 15 (1.7) | 1.0 |
| Enteric | 1 (0.1) | 0 | 1 (0.1) | 1.0 |

Abbreviation: IMA, invasive mucinous adenocarcinoma.

### *Expanded Statistical Analysis of 900 Individual Adenocarcinoma Components*

Of the 629 patients who underwent surgery in 2010, 408 were included in an expanded statistical analysis. Among these patients, 20 were positive for *EML4-ALK* fusions. Of the 45 adenocarcinoma components quantitatively diagnosed in FFPE tissue sections from these 20 patients, 34 samples were identified as positive and 11 as negative for *ALK* fusions from nine patients with intratumoral heterogeneity (Table 1). In the expanded statistical analysis, *ALK* fusions were significantly more common in LADC with mucin production ($P < .001$). Although *ALK* fusions were not significantly associated with an acinar pattern, they were positively associated with a micropapillary pattern and negatively associated with a lepidic pattern (Table 2 ). In addition, the histopathologic characteristics associated with *ALK* fusions/*EGFR* mutations in the 629 LADC patients are listed in Table 3. There was no significant difference in overall survival among the adenocarcinoma subtype groups ($P = .075$; Appendix Fig A3, online only).

## DISCUSSION

Previous studies have demonstrated the intratumoral heterogeneity of either the molecular features or the pathologic features of LADC. However, few studies have focused on the relationship between the two. Therefore, we investigated the potential histologic relevance of the molecular features of LADC, to explore the possible impact of intratumoral heterogeneity on the association between molecular and pathologic features.

A striking finding of our study is the identification of intratumoral genetic heterogeneity in LADC that harbors driver coalterations. The coexistence of *ALK* fusions with *EGFR* mutations was identified in two patients, which provided an incidence rate of 0.3%. Because it is unclear whether *ALK* fusions and *EGFR* mutations coexist in the same tumor cell or in different tumor cells, we used LCM to capture pure tumor cells within both the same and different growth patterns. With this methodology, we observed that *ALK* fusions did not concomitantly coexist with *EGFR* mutations in all tumor cells. Tumor cells that harbored either oncogenic driver were also detected

  

**Table 3.** Clinicopathologic Characteristics of the 629 Patients With LADC

| Characteristic | No. (%) of Patients | | | | | | |
|---|---|---|---|---|---|---|---|
| | | *EML4-ALK* Fusion | | | *EGFR* Mutation | | |
| | All (N= 629) | Positive (n = 30) | Negative (n = 599) | *P* | Positive (n = 364) | Negative (n = 265) | *P* |
| Age, years | | | | | | | |
| Median (range) | 59 (27-82) | 53 (37-78) | 60 (27-82) | | 60 (28-80) | 59 (27-82) | |
| < 65 | 437 (69.5) | 26 (86.7) | 411 (68.6) | .036 | 253 (69.5) | 184 (69.4) | .985 |
| ≥ 65 | 192 (30.5) | 4 (13.3) | 188 (31.4) | | 111 (30.5) | 81 (30.6) | |
| Sex | | | | .635 | | | < .001 |
| Male | 278 (44.2) | 12 (40.0) | 266 (44.4) | | 123 (33.8) | 155 (58.5) | |
| Female | 351 (55.8) | 18 (60.0) | 333 (55.6) | | 241 (66.2) | 110 (41.5) | |
| Smoking history | | | | | | | |
| Never | 468 (74.4) | 23 (76.7) | 445 (74.3) | .865* | 301 (82.7) | 167 (63.0) | < .001* |
| Light smoker (< 10 PY) | 6 (1.0) | 0 | 6 (1.0) | | 5 (1.4) | 1 (0.4) | |
| Smoker (≥ 10 PY) | 155 (24.6) | 7 (23.3) | 148 (24.7) | | 58 (15.9) | 97 (36.6) | |
| Stage | | | | .081† | | | .851† |
| I | 349 (55.5) | 14 (46.7) | 345 (56.0) | | 209 (57.4) | 140 (52.8) | |
| II | 59 (9.4) | 1 (3.3) | 60 (9.7) | | 26 (7.1) | 33 (12.5) | |
| III | 179 (28.5) | 14 (46.7) | 168 (27.3) | | 102 (28.0) | 77 (29.1) | |
| IV | 42 (6.7) | 1 (3.3) | 43 (7.0) | | 27 (7.4) | 15 (5.7) | |
| Histologic subtype | | | | | | | |
| AIS | 6 (1.0) | 0 | 6 (1.0) | | 2 (0.5) | 4 (1.5) | |
| MIA | 5 (0.8) | 0 | 5 (0.8) | | 4 (1.1) | 1 (0.4) | |
| Lepidic | 39 (6.2) | 0 | 39 (6.5) | .247 | 25 (6.9) | 14 (5.3) | .416 |
| Acinar | 309 (49.1) | 20 (66.7) | 289 (48.2) | .049 | 176 (48.4) | 133 (50.2) | .649 |
| Papillary | 198 (31.5) | 5 (16.7) | 193 (32.2) | .073 | 136 (37.4) | 62 (23.4) | < .001 |
| Micropapillary | 21 (3.3) | 3 (10.0) | 18 (3.0) | .073 | 8 (2.2) | 13 (4.9) | .062 |
| Solid | 25 (4.0) | 1 (3.3) | 24 (4.0) | 1.0 | 8 (2.2) | 17 (6.4) | .008 |
| IMA | 24 (3.8) | 1 (3.3) | 23 (3.8) | 1.0 | 4 (1.1) | 20 (7.5) | < .001 |
| Enteric | 2 (0.3) | 0 | 2 (0.3) | | 1 (0.3) | 1 (0.4) | |
| Mucin production | | | | < .001 | | | < .001 |
| Yes | 148 (23.5) | 15 (50.0) | 133 (22.2) | | 63 (17.3) | 85 (32.1) | |
| No | 481 (76.5) | 15 (50.0) | 466 (77.8) | | 301 (82.7) | 180 (67.9) | |
| TTF1 | | | | .002 | | | < .001 |
| Yes | 564 (89.7) | 21 (70.0) | 543 (90.7) | | 340 (93.4) | 224 (84.5) | |
| No | 65 (10.3) | 9 (30.0) | 56 (9.3) | | 24 (6.6) | 41 (15.5) | |
| LADC component | | | | | | | |
| 1 | 116 (18.4) | 3 (10.0) | 113 (18.9) | | | | |
| ≥ 2 | 513 (81.6) | 27 (90.0) | 486 (81.1) | | | | |

Abbreviations: AIS, adenocarcinoma in situ; IMA, invasive mucinous adenocarcinoma; LADC, lung adenocarcinoma; MIA, minimally invasive adenocarcinoma; PY, pack-years; TTF-1, thyroid transcription factor-1.
*Never/light smokers versus smokers.
†Stage I and II versus stage III and IV.

in the two patients with *ALK*/*EGFR* coaltered tumors. Of interest, one of the selected areas with an acinar pattern was identified as negative for both *ALK* fusions and *EGFR* mutations by RT-PCR and ARMS assays. To exclude the possibility of a third oncogene, we performed targeted DNA sequencing by using a capture-based targeted sequencing panel. We did not identify a third oncogene, and we also observe a difference in the driver status among spatially separated tumor areas. In particular, the relative abundance of the two altered genes in the same tumor areas was different, which suggested that tumor cells with a single driver or without driver may also exist in LADC with dual drivers (Fig 2B). It therefore seems reasonable to infer that the oncogenic driver profile may not be the same in all tumor cells within the same primary tumor because of the genetic intratumoral heterogeneity of LADC. Yang et al[23] considered that *ALK* fusion proteins and *EGFR* mutant proteins coexist in the same tumor cells. However, they drew this conclusion just by the detection of protein expression in serial sections, rather than in the same section, of FFPE tissue tumor samples. Thus, what they observed was not the same, but rather was several adjacent tumor cells. In addition, they also found a difference in protein expression levels between phospho-EGFR and phospho-ALK proteins in *ALK*/*EGFR* coaltered tumors, which suggested that coaltered driver genes may not coexist in all tumor cells.[23]

Because we fully recognized and controlled morphologic heterogeneity of LADC in our study, we found by using LCM that the molecular features of tumor cells in the same adenocarcinoma component were not all the same, especially in two patients with *ALK*/*EGFR* coaltered tumors. For these two patients, selected areas in the same growth pattern were found to have different statuses for *EGFR* mutations or *ALK* fusions. We also observed similar results by using targeted DNA sequencing in two *ALK*-positive patients. Although Tomonaga et al[24] found that

intratumoral heterogeneity of *EGFR* mutations was associated with the distribution of histologic subtypes in mixed-type LADCs, we considered that intratumoral heterogeneity of *EGFR* mutations also exists in the same histologic subtypes of LADC. Therefore, we speculate that clone evolution, instead of only histologic heterogeneity, may be mainly responsible for molecular intratumoral heterogeneity of LADC.

The findings of intratumoral heterogeneity in both *ALK*-rearranged and *ALK*/*EGFR* coaltered LADC may be explained by Darwinian-like clonal evolutionary dynamics and the resulting complex clonal architecture of LADC. Previous studies have shown that a substantial proportion of malignant tumors have a multiclonal signature.[25] Chiari et al[26] reported that a patient achieved a long-term response to crizotinib after acquiring resistance to *EGFR*-tyrosine kinase inhibitors (TKIs). Of interest, the 2009 biopsy specimen from this patient was confirmed positive for *ALK* translocation and wild-type *EGFR*, although an *EGFR* L858R mutation was identified in the 2004 biopsy specimen. The authors inferred that the tumor harbored dual altered driver genes in different neoplastic clones at the first diagnosis and that *ALK*-rearranged clones were selected by the TKI therapy.[26] Certainly, the diagnosis of driver status might be affected by the sensitivity of test methods. Furthermore, a study to investigate the mechanism of resistance to crizotinib by culturing primary cell lines derived from patients revealed that alternate oncogenes, including *KRAS* or *EGFR*, exist in separate subclonal populations that lack an *ALK* gene rearrangement.[27] Resistance to TKIs is considered one of the known unknowns of cancer; therapy selection may make tumors become more heterogeneous for intratumoral genetic heterogeneity, which may be the major reason for resistance to TKIs.[28] The complex dynamics of clonal evolution could produce unique and unpredictable patterns of clonal architecture that are spatially and temporally heterogeneous.[29,30] Clonal evolution that underlies tumor progression probably proceeds in a branching, rather than in a linear, manner, which might lead to substantial clonal diversity that additionally contributes to genetic heterogeneity within tumors.[30] Importantly, our findings may provide a rationale for differently treating patients with LADC that harbors dual drivers. It seems reasonable to treat patients who do harbor dual drivers with two different targeted inhibitors if the oncogenic drivers of tumor cells within the same primary tumor are not all the same.

In addition, any analyses that were based on a pathologic diagnosis of the predominant component in LADC did not adequately determine the pathologic features of *ALK* rearrangements. In our study, only 18.4% of patients (116 of 629) showed a pure single pattern, which was consistent with 80% to 90% of surgically resected adenocarcinomas that showed more than one growth pattern.[9] Therefore, we performed a statistical analysis in an expanded population of 900 individual adenocarcinoma components diagnosed in FFPE tissue sections. This analysis appeared to show that *ALK* fusions were significantly more common in LADCs with a micropapillary pattern and less common in those with a lepidic pattern. However, given the potential impact of genetic intratumoral heterogeneity on the histologic features of *ALK* fusions, especially in LADC that contains greater than one histologic subtype, the relationship may be more complex than it looks.

In conclusion, because of the high intratumoral heterogeneity of both the molecular and the histopathologic features of LADC, the correlation of the two seems to be of less significance for clinical diagnosis and treatment. In addition, our study also observed the intratumoral heterogeneity of oncogenic drivers in *ALK*/*EGFR* coaltered samples. Intratumoral heterogeneity of molecular oncogenic drivers in LADC should be taken seriously, because they can hinder accurate diagnosis and selection of the most appropriate treatment in clinical practice. Additional studies are needed to explore the possible mechanisms.

## AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at www.jco.org.

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## AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

**Intratumoral Heterogeneity of *ALK*-Rearranged and *ALK*/*EGFR* Coaltered Lung Adenocarcinoma**

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## Appendix

### Inclusion Criteria

All participating patients were required to meet the following inclusion criteria: age 18 years or older; histologically confirmed lung adenocarcinoma; sufficient formalin-fixed, paraffin-embedded tissue available for *EGFR* mutation and *ALK* fusion screening and validation; demographic data available for analysis that included age, sex, smoking status, histologic type, disease stage, and provision of written informed consent. Patients did not receive preoperative systemic or radiation therapy.

**Table A1.** *ALK* Fusions Screened in the Study

| Fusion No. | COSMIC ID | *EML4* Exon | Breakpoint | Insert (bp) | *ALK* Exon | Breakpoint |
|---|---|---|---|---|---|---|
| 1 | COSF463 | 13 | 1751 | — | 20 | 4080 |
| 2 | COSF489 | 13 | 1751 + 447 | — | 20 | 4080-161 |
| 3 | COSF1063 | 13 | 1751 | 69 | 20 | 4080-69 |
| 4 | COSF462 | 13 | 1751 + (3600) | — | 20 | 4080-297 |
| 5 | COSF410 | 13 | 1751 + 1485 | — | 20 | 4080-1254 |
| 6 | COSF414 | 13 | 1751 + 2575 | — | 20 | 4080-203 |
| 7 | COSF474 | 6 | 929 + 220 | — | 20 | 4080 |
| 8 | COSF734 | 6 | 929 | — | 20 | 4080 |
| 9 | COSF476 | 6 | 929 + (7320) | 33 | 20 | 4080 |
| 10 | COSF493 | 6 | 929 + 805 | — | 20 | 4080-115 |
| 11 | COSF465 | 20 | 2504 | — | 20 | 4080 |
| 12 | COSF490 | 20 | 2504 + 182 | — | 20 | 4080-67 |
| 13 | COSF731 | 20 | 2504 | 18 | 20 | 4080-18 |
| 14 | COSF464 | 20 | 2504 + 545 | — | 20 | 4080-232 |
| 15 | COSF488 | 18 | 2318 + 654 | — | 20 | 4080-172 |
| 16 | COSF480 | 2 | 470 | — | 20 | 4080-117 |
| 17 | COSF480 | 2 | 470 | 117 | 20 | 4080-117 |

Abbreviation: COSMIC, Catalog of somatic mutations in cancer.

**Table A2.** Forty-Seven Genes Included in the Capture-Based Targeted DNA Sequencing Panel

| | Targeted DNA | |
|---|---|---|
| Gene No. | Whole Exon | Intron |
| 1 | *AKT1* | |
| 2 | *ALK* | *ALK* |
| 3 | *APC* | |
| 4 | *ATM* | |
| 5 | *AURKA* | |
| 6 | *BIM* | |
| 7 | *BRAF* | |
| 8 | *CCND1* | |
| 9 | *CDK4* | |
| 10 | *CDK6* | |
| 11 | *CDKN2A* | |
| 12 | *CTNNB1* | |
| 13 | *DDR2* | |
| 14 | *EGFR* | |
| 15 | *ERBB2* | |
| 16 | *ERBB4* | |
| 17 | *FGFR1* | |
| 18 | *FGFR2* | |
| 19 | *FGFR3* | |
| 20 | *FLT3* | |
| 21 | *IGF1R* | |
| 22 | *JAK2* | |
| 23 | *KDR* | |
| 24 | *KIT* | |
| 25 | *KRAS* | |
| 26 | *MAP2K1* | |
| 27 | *MDM2* | |
| 28 | *MET* | |
| 29 | *MTOR* | |
| 30 | *NF1* | |
| 31 | *NOTCH1* | |
| 32 | *NRAS* | |
| 33 | *NRG1* | |
| 34 | *NTRK1* | |
| 35 | *NTRK2* | |
| 36 | *NTRK3* | |
| 37 | *PDGFRA* | |
| 38 | *PIK3CA* | |
| 39 | *PTEN* | |
| 40 | *RB1* | |
| 41 | *RET* | *RET* |
| 42 | *ROS1* | *ROS1* |
| 43 | *SMO* | |
| 44 | *STK11* | |
| 45 | *TP53* | |
| 46 | *TSC1* | |
| 47 | *TSC2* | |

**Table A3.** Pathologic Subtype and Driver Status of Five Patients With *EGFR*-Mutated Adenocarcinoma and Genetic Intratumoral Heterogeneity

| Isolated Area | LADC Subtype | Mucin Production | *EGFR* Mutation |
|---|---|---|---|
| LUC21 | | | |
| A | Acinar | No | Wild type |
| B | Acinar | No | Wild type |
| C | Papillary | No | Wild type |
| D | Papillary | No | L858R |
| LUC22 | | | |
| A | Acinar | Yes | Wild type |
| B | Acinar | Yes | Wild type |
| C | Acinar | Yes | L858R |
| D | Acinar | Yes | L858R |
| LUC23 | | | |
| A | Lepidic | No | Wild type |
| B | Acinar | No | L858R |
| C | Acinar | Yes | 19del/L858R |
| LUC24 | | | |
| A | Acinar | No | L858R |
| B | Papillary | No | Wild type |
| C | Papillary | No | L858R |
| D | Papillary | No | L858R |
| LUC25 | | | |
| A | Acinar | No | Wild type |
| B | Acinar | No | Wild type |
| C | Acinar | No | L858R |

Abbreviation: LUC, lung cancer sample.

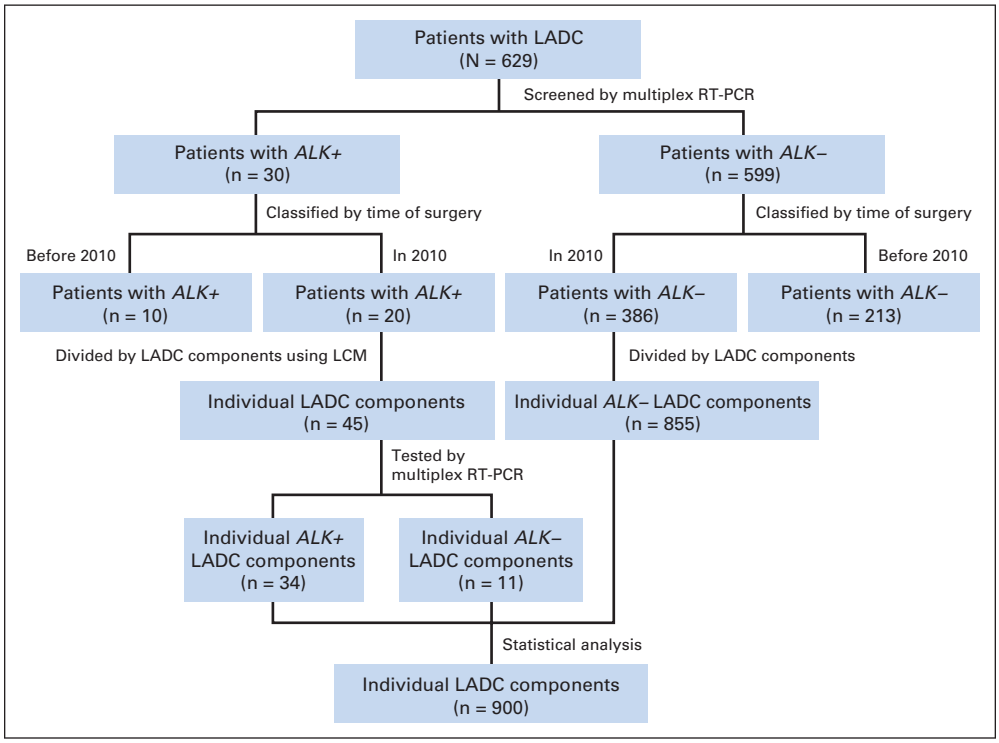


**Fig A1.** Study flow chart. LADC, lung adenocarcinoma; LCM, laser-capture microdissection; RT-PCR, reverse-transcriptase polymerase chain reaction.

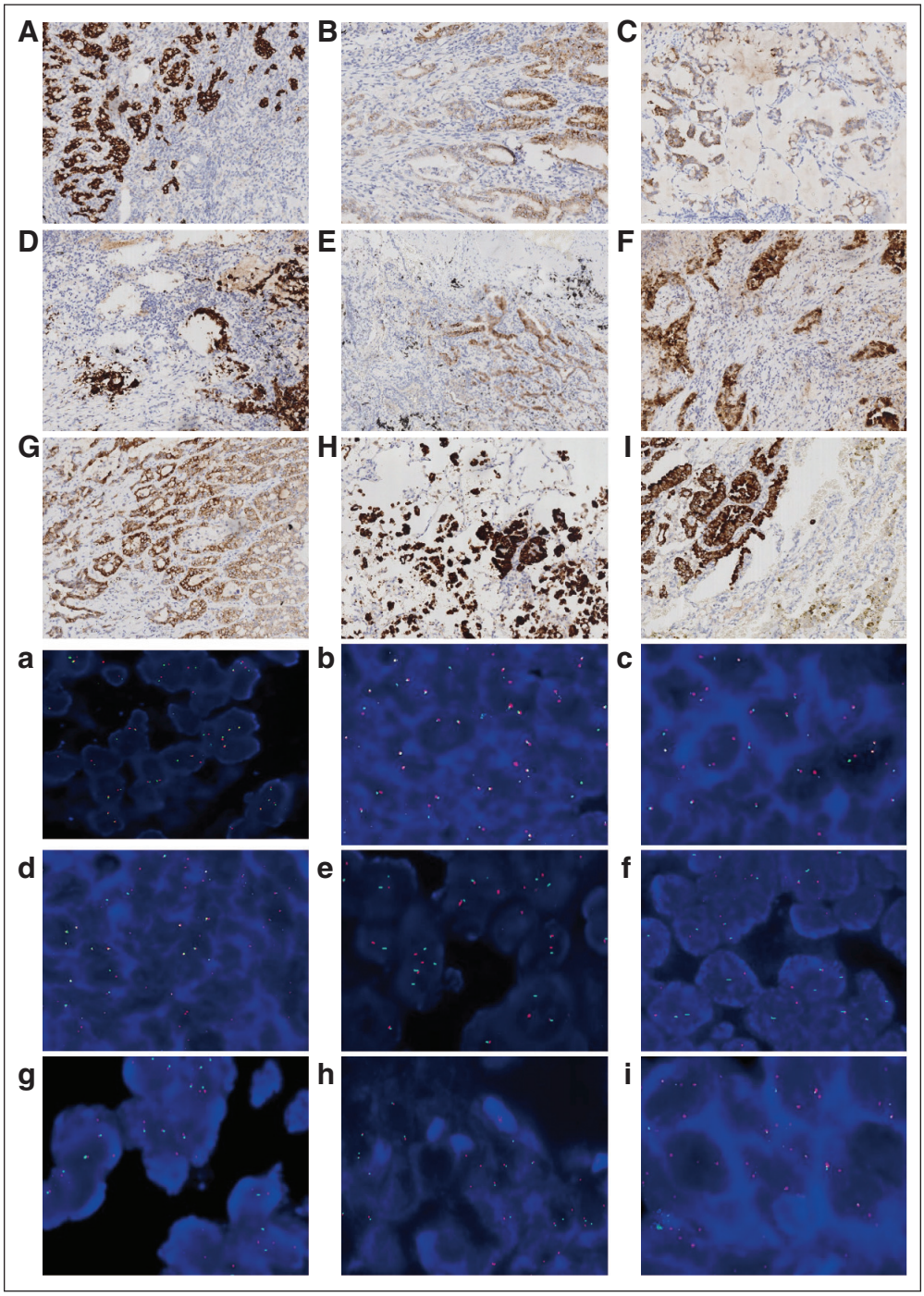


**Fig A2.** *ALK* rearrangement confirmed by (A-I) Ventana immunohistochemistry (IHC) and (a-i) fluorescent in situ hybridization (FISH) in nine patients with lung adenocarcinoma and genetic heterogeneity. Both Ventana IHC stain–negative and break-apart FISH probe signal–negative regions were identified in these nine patients.

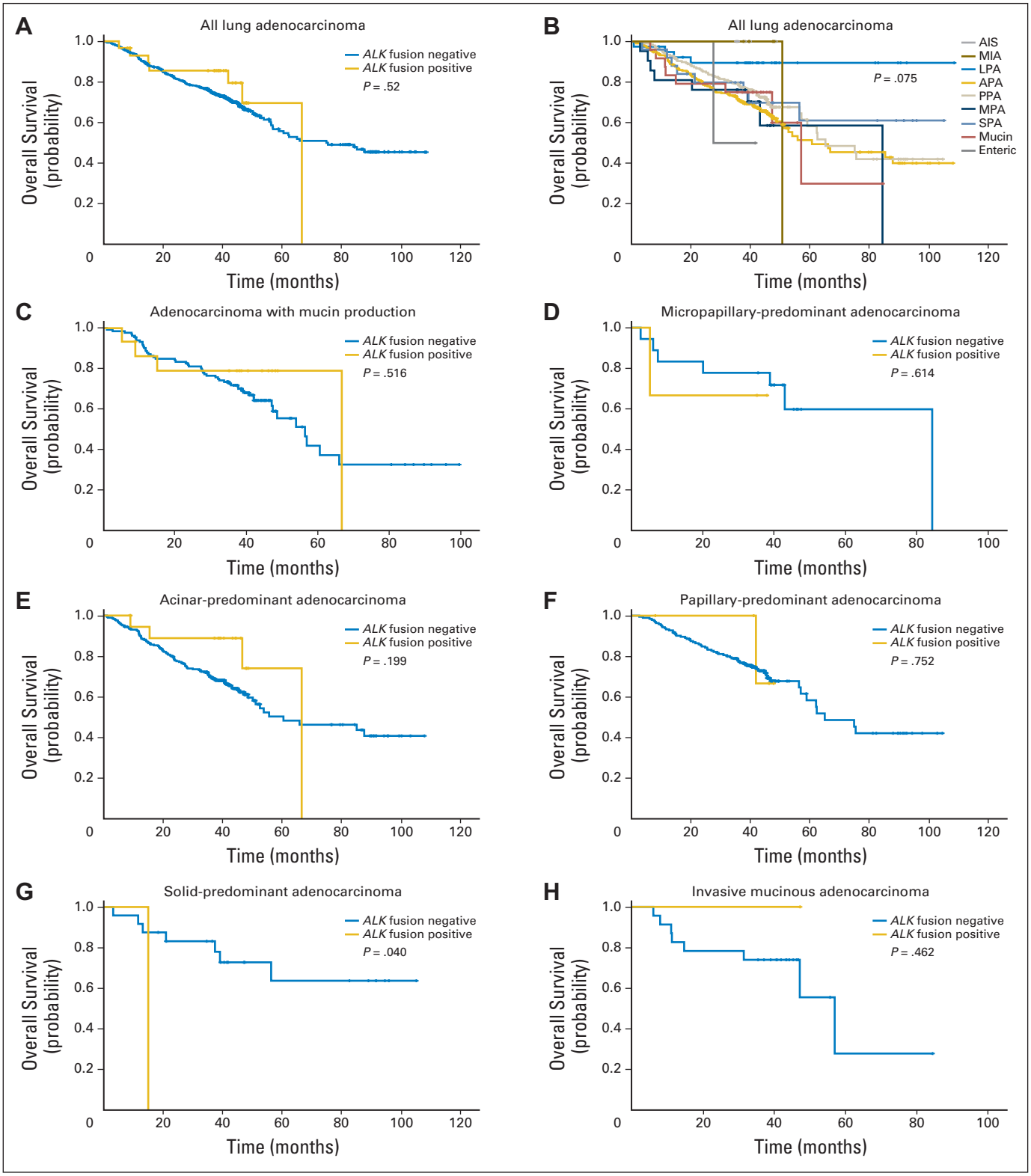


**Fig A3.** Kaplan-Meier curves of overall survival (OS). (A) Comparison of OS between *ALK*-negative and *ALK*-positive groups in the 629 patients with lung adenocarcinoma. (B) Comparison of OS between groups with different adenocarcinoma subtypes in the 629 patients with lung adenocarcinoma. (C) Comparison of OS between *ALK*-negative and *ALK*-positive groups in 148 adenocarcinomas with mucin production. (D) Comparison of OS between *ALK*-negative and *ALK*-positive groups in 21 micropapillary-predominant adenocarcinomas. (E) Comparison of OS between *ALK*-negative and *ALK*-positive groups in 309 acinar-predominant adenocarcinomas. (F) Comparison of OS between *ALK*-negative and *ALK*-positive groups in 198 papillary-predominant adenocarcinomas. (G) Comparison of OS between *ALK*-negative and *ALK*-positive groups in 25 solid-predominant adenocarcinomas. (H) Comparison of OS between *ALK*-negative and *ALK*-positive groups in 24 invasive mucinous adenocarcinomas. AIS, adenocarcinoma in situ; APA, acinar-predominant adenocarcinoma; Enteric, enteric adenocarcinoma; LPA, lepidic-predominant adenocarcinoma; MIA, minimally invasive adenocarcinoma; MPA, micropapillary-predominant adenocarcinoma; Mucin, adenocarcinoma with mucin production; PPA, papillary-predominant adenocarcinoma; SPA, solid-predominant adenocarcinoma.